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(54) Title: STARCH BRANCHING ENZYME II OF POTATO

(57) Abstract

The present invention relates to an amino acid sequence of second starch branching enzyme (SBE II) of potato and a fragment thereof as well as to the corresponding isolated DNA sequences. Furthermore, the invention relates to vectors comprising such an isolated DNA sequence, to processes for production of transgenic potatoes, and to the use of said potatoes for the production of starch. The starch obtained will show a changed pattern of branching of amylopectin as well as a changed amylose/amylopectin ratio.

# STARCH BRANCHING ENZYME II OF POTATO

The present invention relates to a novel starch branching enzyme of potato. More specifically, the present invention relates to an amino acid sequence of a second starch branching enzyme (SBE II) of potato and a fragment thereof as well as their corresponding DNA sequences. Furthermore, the invention relates to vectors comprising such DNA sequences, to processes for production of transgenic potatoes, and to the use of said potatoes for the production of starch.

Starch is a complex mixture of different molecule forms differing in degree of polymerization and branching of the glucose chains. Starch consists of amylose and amylopectin, whereby the amylose consists of an essentially linear α-1,4-glucan and amylopectin consists of α-1,4-glucans connected to each other via α-1,6-linkages and, thus, forming a branched polyglucan. Thus, starch is not a uniform raw material.

Starch is synthesized via at least three enzymatic reactions in which ADP glucose phosphorylase (EC 2.7.7.27), starch synthase (EC 2.4.1.21) and starch branching enzyme (EC 2.4.1.18) are involved. Starch branching enzyme (SBE, also called Q-enzyme) is believed to have two different enzymatic activities. It catalyzes both the hydrolysis of α-1,4-glucosidic bonds and the formation of α-1,6-glucosidic bonds during synthesis of the branched component in starch, i.e. amylopectin.

Plant starch is a valuable source of renewable raw material used in, for example, the chemical industry (Visser and Jacobsen, 1993). However, the quality of the starch has to meet the demands of the processing industry wherein uniformity of structure is an important criterion. For industrial application there is a need of plants only containing amylose starch and plants only containing amylopectin starch, respectively.

Processes for altering the amylose/amylopectin ratio in starch have already been proposed. For example, in WO95/04826 there is described DNA sequences encoding debranching enzymes with the ability to reduce or increase the degree of branching of amylopectin in transgenic plants, e.g. potatoes.

In WO92/14827 plasmids are described having DNA sequences that after insertion into the genome of the plants cause changes in the carbohydrate concentration and the carbohydrate composition in regenerated plants. These changes can be obtained from a sequence of a branching enzyme that is located on these plasmids. This branching enzyme is proposed to alter the amylose/amylopectin ratio in starch of the plants, especially in commercially used plants.

WO92/14827 describes the only hitherto known starch branching enzyme in potato and within the art it is not known whether other starch branching enzymes are involved in the synthesis of branched starch of potato.

In Mol Gen Genet (1991) 225:289-296, Visser et al., there is described inhibition of the expression of the gene for granule-bound starch synthase in potato by antisense constructs. Inhibition of the enzyme in potato tuber starch was up to 100% in which case amylose-free starch was provided.

However, the prior known methods for inhibiting amylopectin have not been that successful and, therefore, alternative methods for inhibiting amylopectin are still highly desirable (Müller-Röber and Koßmann, 1994; Martin and Smith, 1995).

The object of the present invention is to enable altering the degree of amylopectin branching and the amylopectin/amylose ratio in potato starch.

According to the present invention this object is achieved by providing a novel isolated DNA sequence encoding a second starch branching enzyme, SBE II, and

fragments thereof, which after insertion into the genome of the plants cause changes in said branching degree and ratio in regenerated plants.

Within the scope of the present invention there is also included the amino acid sequence of SBE II and fragments thereof.

Also variants of the above DNA sequence resulting from the degeneracy of the genetic code are encompassed.

The novel DNA sequence encoding SBEII, comprising 3074 nucleotides, as well as the corresponding amino acid sequence comprising 878 amino acids, are shown in SEQ ID No. 1. One 1393 nucleotides long fragment of the above DNA sequence, corresponding to nucleotides 1007 to 2399 of the DNA sequence in SEQ ID No. 1, as well as the corresponding amino acid sequence comprising 464 amino acids, are shown in SEQ ID No. 2.

Furthermore, there are provided vectors comprising said isolated DNA-sequences and regulatory elements active in potato. The DNA sequences may be inserted in the sense or antisense (reversed) orientation in the vectors in relation to a promoter immediately upstream from the DNA sequence.

Also there is provided a process for the production of transgenic potatoes with a reduced degree of branching of amylopectin starch, comprising the following steps:
a) transfer and incorporation of a vector according to the invention into the genome of a potato cell, and
b) regeneration of intact, whole plants from the transformed cells.

Finally, the invention provides the use of said transgenic potatoes for the production of starch.

The invention will be described in more detail below in association with an experimental part and the accompanying drawings, in which

Fig. 1 shows SDS polyacrylamide electrophoresis of proteins extracted from starch of normal potato (lane A)

and transgenic potato (lane B). Excised protein bands are marked with arrows. Lane M: Molecular weight marker proteins (kDa).

Fig. 2 shows 4 peptide sequences derived from digested proteins from potato tuber starch.

EXPERIMENTAL PART

*Isolation of starch from potato tubers*

Potato plants (*Solanum tuberosum*) were grown in the field. Peeled tubers from either cv. Early Puritan or from a transgenic potato line essentially lacking granule-bound starch synthase I (Svalöf Weibull AB, international application number PCT/SE91/00892), were homogenized at 4°C in a fruit juicer. To the "juice fraction", which contained a large fraction of the starch, was immediately added Tris-HCl, pH 7.5, to 50 mM, Na-dithionite to 30 mM and ethylenedinitrilotetraacetic acid (EDTA) to 10 mM. The starch granules were allowed to sediment for 30 min and washed 4x with 10 bed volumes of washing buffer (50 mM Tris-HCl, pH 7.5, 10 mM EDTA). The starch, which was left on the bench at +4°C for 30 min to sediment between every wash, was finally washed with 3 x 3 bed volumes of acetone, air dried over night, and stored at -20°C.

*Extraction of proteins from tuber starch*

Stored starch (20 g) was continuously mixed with 200 ml extraction buffer (50 mM Tris-HCl, pH 7.5, 2% (w/v) sodium dodecyl sulfate (SDS), 5 mM EDTA) by aspiration with a pipette at 85°C until the starch was gelatinized. The samples were then frozen at -70°C for 1 hour. After thawing at 50°C, the samples were centrifuged for 20 min at 12,000xg at 10°C. The supernatants were collected and re-centrifuged at 3,000xg for 15 min. The final supernatants were filtered through 0.45 μ filters and 2.25 volumes of ice-cold acetone were added. After 30 min incubation at 4°C, the protein precipitates were collected by centrifugation (3,000xg for 30 min at 4°C), and

dissolved in 50 mM Tris-HCl, pH 7.5. An aliquot of each preparation was analyzed by SDS poly-acrylamide gel electrophoresis according to Laemmli (1970) (Fig. 1). The proteins in the remaining portions of the preparations were concentrated by precipitation with trichloroacetic acid (10%) and the proteins were separated on an 8% SDS polyacrylamide gel Laemmli, (1970). The proteins in the gel were stained with Coomassie Brilliant Blue R-250 (0.2% in 20% methanol, 0.5% acetic acid, 79.5% $H_2O$).

*In gel digestion and sequencing of peptides*

The stained bands marked with arrows in Fig. 1 corresponding to an apparent molecular weight of about 100 kDa were excised and washed twice with 0.2M $NH_4HCO_3$ in 50% acetonitrile under continuous stirring at 35°C for 20 min. After each washing, the liquid was removed and the gel pieces were allowed to dry by evaporation in a fume hood. The completely dried gel pieces were then separately placed on parafilm and 2 µl of 0.2M $NH_4CO_3$, 0.02% Tween-20 were added. Modified trypsin (Promega, Madison, WI, USA) (0.25 µg in 2 µl) was sucked into the gel pieces whereafter 0.2M $NH_4CO_3$ was added in 5 µl portions until they had resumed their original sizes. The gel slices were further divided into three pieces and transferred to an Eppendorf tube. 0.2M $NH_4CO_3$ (200 µl) was added and the proteins contained in the gel pieces were digested over night at 37°C (Rosenfeld et al. 1992). After completed digestion, trifluoroacetic acid was added to 1% and the supernatants removed and saved. The gel pieces were further extracted twice with 60% acetonitrile, 0.1% trifluoroacetic acid (200 µl) under continuous shaking at 37°C for 20 min. The two supernatants from these extractions were combined with the first supernatant. The gel pieces were finally washed with 60% acetonitrile, 0.1% trifluoroacetic acid, 0.02% Tween-20 (200 µl). Also these supernatants were combined with the other supernatants and the volume was reduced to 50 µl by evaporation. The

extracted peptides were separated on a SMART chromatography system (Pharmacia, Uppsala, Sweden) equipped with a µRPC C2/C18 SC2.1/10 column. Peptides were eluted with a gradient of 0 - 60% acetonitrile in water/0.1% trifluoroacetic acid over 60 min with a flow rate of 100 µl/min. Peptides were sequenced either on an Applied Biosystems 470A gas phase sequenator with an on line PTH-amino acid analyzer (120A) or on a model 476A according to the instructions of the manufacturer (Applied Biosystems, Foster City, CA, USA).

Four of the peptides sequenced gave easily interpretable sequences (Fig. 2). A data base search revealed that these four peptides displayed similarity to starch branching enzymes and interestingly, the peptides were more related to starch branching enzyme II from other plant species than to starch branching enzyme I from potato.

*Construction of oligonucleotides encoding peptides 1 and 2.*

Degenerated oligonucleotides encoding peptide 1 and peptide 2 were synthesized as forward and reverse primers, respectively:

Oligonucleotide 1: 5'-gtaaaacgacggccagt-TTYGGNGTNTGGGARATHTT-3' (Residues 2 to 8 of peptide 1)

Oligonucleotide 2: 5'-aattaaccctcactaaaggg-CKRTCRAAYTCYTGIARNCC-3' (Residues 2 to 8 of peptide 2, reversed strand)

wherein

H is A, C or T, I is inosine; K is G or T; N is A, C, G or T; R is A or G; Y is C or T; bases in lower case were added as tag sequences.

*Purification of mRNA from potato tuber, synthesis of cDNA and PCR amplification of a cDNA fragment corresponding to potato starch branching enzyme II.*

Total RNA from mature potato tubers (*S. tuberosum* cv. Amanda) was isolated as described (Logemann et al. 1987). First strand cDNA was synthesized using 2 µg of total RNA and 60 pmol of oligo-$dT_{30}$ as downstream primer. The primer was annealed to the polyA of the mRNA at 60°C for 5 min. The extension of the cDNA was performed according to the technical manual of the manufacturer using the Riboclone® cDNA Synthesis System M-MLV (H-)(Promega).

cDNA encoding the novel starch branching enzyme II according to the invention was amplified in a Perkin-Elmer GeneAmp® 9600 PCR thermocycler (Perkin-Elmer Cetus Instruments, CT, USA) using the two degenerate primers designed from the peptides 1 and 2 (see above) under the following conditions: 1 mM dNTP, 1 µM of each primer and an alicot of the cDNA described above in a total reaction volume of 20 µl with 1x AmpliTaq® buffer and 0,8 U AmpliTaq® (Perkin-Elmer Cetus). The cycling conditions were: 96°C for 1', 80°C while the enzyme was added as a hotstart (approximately 15'), an unintended drop to 25°C, five cycles of 94°C for 30", 45°C for 1', ramp to 72°C for 1' and 72°C for 2', and 30 cycles of 94°C for 5", 45°C for 30", and 72°C for (2'+2" per cycle) and completed with 72°C for 10' prior to chilling to 4°C.

A sample of this reaction (0.1 µl) was reamplified using the cycling conditions: 96°C for 1', 80°C while the enzyme was added as a hotstart (approximately 5'), five cycles of 94°C for 20'', 45°C for 1', and 72°C for 2', and 25 cycles of 94°C for 5'', 45°C for 30'', and 72°C for (2' + 2'' per cycle) and completed with 72°C for 10' prior to chilling to 4°C. After completion of the PCR amplification, the reaction was loaded on a 1.5% Seakem agarose gel (FMC Bioproducts, Rockland, ME, USA). After electrophoresis and staining with ethidium bromide a major

band with an apparent size of 1500 bp was excised and the fragment was eluted by shaking in water (200 µl) for 1 h. This fragment was used as template in sequencing reactions after reamplification using primers corresponding to the tag sequences (in oligonucleotides 1 and 2), purification by agarose gel electrophoresis as above and extraction from the gel using the Qiaex® gel extraction kit according to the manufacturer's instructions (DIAGEN GmbH, Hilden, Germany). The sequencing reactions were done using the DyeDeoxy® Terminator Cycle Sequencing kits (Perkin-Elmer Cetus Instruments) using tag sequences and internal primers. The sequencing reaction were analyzed on an Applied Biosystems 373A DNA sequencer according to the manufacturer's protocols. The sequence was edited and comprised 1393 bp.

To complete the determination of the sequence of starch branching enzyme II, the 5' and 3' ends of the full length cDNA were amplified from the same total RNA as above using rapid amplification of cDNA ends, RACE, methodology with specific primers from the 1393 bp sequence. In the 3' end amplification, an oligo $T_{20}G$ primer was used against the poly A tail and in the 5' end, the 5'/3' RACE kit from Boehringer Mannheim (Cat. No. 1734792) was used. The fragments from these amplifications were sequenced in the same way as above using internal and end primers. The sequences from the two ends were aligned together with the 1393 base pairs to give a composite full length cDNA sequence. Primers were designed from this sequence to amplify the whole coding region in one part. Partial sequencing of the amplified coding cDNA confirmed the presence of a cDNA corresponding to the composite sequence. The full length cDNA is 3074 bp and the translated sequence comprises 878 amino acids. The mature protein comprises 830 amino acids.

Comparisons of the consensus sequence with the EMBL and GenBank databases showed 68% identity to potato starch

branching enzyme I and about 60% identity to starch branching enzyme II from other plant species. The present inventors therefore denote the enzyme encoded by the new branching enzyme sequence potato starch branching enzyme II.

*Transformation of potato plants*

The isolated full length cDNA of potato starch branching enzyme II and other functionally active fragments in the range of 50-3 074 bp are cloned in reverse orientation behind promoters active in potato tubers. By the term "functionally active" is meant fragments that will affect the amylose/amylopectin ratio in potato starch. The DNA and amino acid sequence of SBE II according to the invention as well as one fragment of the DNA and corresponding amino acid sequence are shown in SEQ ID No. 1 and 2, respectively.

The promoters are selected from, for example, the patatin promoter, the promoter from the potato granule-bound starch synthase I gene or promoters isolated from potato starch branching enzymes I and II genes.

The constructs are cloned by techniques known in the art either in a binary Ti-plasmid vector suitable for transformation of potato mediated by *Agrobacterium tumefaciens*, or in a vector suitable for direct transformation using ballistic techniques or electroporation. It is realized that the sense (see below) and antisense constructs must contain all necessary regulatory elements.

Transgenic potato plants transcribe the inverse starch branching enzyme II construct specifically in tubers, leading to antisense inhibition of the enzyme. A reduction and changed pattern of the branching of amylopectin as well as a changed amylose/amylopectin ratio thereby occur in tuber starch.

The antisense construct for potato starch branching enzyme II is also used in combination with antisense

constructs for potato starch branching enzyme I, for potato granule-bound starch synthase II, for potato soluble starch synthases II and III, for potato starch disproportionating enzyme (D-enzyme) or for potato starch debranching enzyme to transform potato to change the degree of branching of amylopectin and the amylose/amylopectin ratio. This gives new and valuable raw material to the starch processing industry.

The full-length cDNA sequence encoding the enzyme is, in different constructs, cloned in sense orientation behind one or more of the promoters mentioned above, and the constructs are transferred into suitable transformation vectors as described above and used for the transformation of potato. Regenerated transformed potato plants will produce an excess of starch branching enzyme II in the tubers leading to an increased degree and changed pattern of branching of amylopectin or to inhibition of transcription of endogenous starch branching enzyme II transcription due to co-suppression, resulting in a decreased branching of amylopectin.

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Visser, R.G.F., Jacobsen, E. (1993) Towards modifying plants for altered starch content and composition. TibTech 11, 63-68.

SEQ ID No. 1

Sequenced molecule: cDNA
Name: beII gene (branching enzyme II) from Solanum tuberosum (potato)
Length of sequence: 3074 bp

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AAACCTCCTC GACTCAGTCT TTGTTTCTCT CTCTCTTCAC GCTTCTCTTG GCGCCTTGAA     60
CTCAGGAATT TGACACTCAG TTAGTTACAC TNCCATCACT TATCAGATCT CTATTTTTTC    120
TCTTAATTCC AACCAAGGAA TGAATAAAAA GATAGATTTG TAAAAACCCT AAGGAGAGAA    180
GAAGAAAG ATG GTG TAT ACA CTC TCT GGA GTT CGT TTT CCT ACT GTT CCA     230
         Met Val Tyr Thr Leu Ser Gly Val Arg Phe Pro Thr Val Pro
                     -45                 -40                 -35

TCA GTG TAC AAA TCT AAT GGA TTC AGC AGT AAT GGT GAT CGG AGG AAT      278
Ser Val Tyr Lys Ser Asn Gly Phe Ser Ser Asn Gly Asp Arg Arg Asn
                -30                 -25                 -20

GCT AAT NTT TCT GTA TTC TTG AAA AAG CAC TCT CTT TCA CGG AAG ATC      326
Ala Asn Xaa Ser Val Phe Leu Lys Lys His Ser Leu Ser Arg Lys Ile
            -15                 -10                  -5

TTG GCT GAA AAG TCT TCT TAC AAT TCC GAA TCC CGA CCT TCT ACA GTT      374
Leu Ala Glu Lys Ser Ser Tyr Asn Ser Glu Ser Arg Pro Ser Thr Val
        1               5                  10

GCA GCA TCG GGG AAA GTC CTT GTG CCT GGA ACC CAG AGT GAT AGC TCC      422
Ala Ala Ser Gly Lys Val Leu Val Pro Gly Thr Gln Ser Asp Ser Ser
 15                 20                  25                  30

TCA TCC TCA ACA GAC CAA TTT GAG TTC ACT GAG ACA TCT CCA GAA AAT      470
Ser Ser Ser Thr Asp Gln Phe Glu Phe Thr Glu Thr Ser Pro Glu Asn
                35                  40                  45

TCC CCA GCA TCA ACT GAT GTA GAT AGT TCA ACA ATG GAA CAC GCT AGC      518
Ser Pro Ala Ser Thr Asp Val Asp Ser Ser Thr Met Glu His Ala Ser
            50                  55                  60

CAG ATT AAA ACT GAG AAC GAT GAC GTT GAG CCG TCA AGT GAT CTT ACA      566
Gln Ile Lys Thr Glu Asn Asp Asp Val Glu Pro Ser Ser Asp Leu Thr
        65                  70                  75

GGA AGT GTT GAA GAG CTG GAT TTT GCT TCA TCA CTA CAA CTA CAA GAA      614
Gly Ser Val Glu Glu Leu Asp Phe Ala Ser Ser Leu Gln Leu Gln Glu
    80                  85                  90

GGT GGT AAA CTG GAG GAG TCT AAA ACA TTA AAT ACT TCT GAA GAG ACA      662
Gly Gly Lys Leu Glu Glu Ser Lys Thr Leu Asn Thr Ser Glu Glu Thr
 95                 100                 105                 110

ATT ATT GAT GAA TCT GAT AGG ATC AGA GAG AGG GGC ATC CCT CCA CCT      710
Ile Ile Asp Glu Ser Asp Arg Ile Arg Glu Arg Gly Ile Pro Pro Pro
                115                 120                 125

GGA CTT GGT CAG AAG ATT TAT GAA ATA GAC CCC CTT TTG ACA AAC TAT      758
Gly Leu Gly Gln Lys Ile Tyr Glu Ile Asp Pro Leu Leu Thr Asn Tyr
            130                 135                 140

CGT CAA CAC CTT GAT TAC AGG TAT TCA CAG TAC AAG AAA CTG AGG GAG      806
Arg Gln His Leu Asp Tyr Arg Tyr Ser Gln Tyr Lys Lys Leu Arg Glu
        145                 150                 155
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GCA ATT GAC AAG TAT GAG GGT GGT TTG GAA GCT TTT TCT CGT GGT TAT     854
Ala Ile Asp Lys Tyr Glu Gly Gly Leu Glu Ala Phe Ser Arg Gly Tyr
    160                 165                 170

GAA AAA ATG GGT TTC ACT CGT AGT GCT ACA GGT ATC ACT TAC CGT GAG     902
Glu Lys Met Gly Phe Thr Arg Ser Ala Thr Gly Ile Thr Tyr Arg Glu
175                 180                 185                 190

TGG GCT CCT GGT GCT CAG TCA GCT GCC CTC ATT GGA GAT TTC AAC AAT     950
Trp Ala Pro Gly Ala Gln Ser Ala Ala Leu Ile Gly Asp Phe Asn Asn
                195                 200                 205

TGG GAT GCA AAT GCT GAC ATT ATG ACT CGG AAT GAA TTT GGT GTC TGG     998
Trp Asp Ala Asn Ala Asp Ile Met Thr Arg Asn Glu Phe Gly Val Trp
            210                 215                 220

GAG ATT TTT CTG CCA AAT AAT GTG GAT GGT TCT CCT GCA ATT CCT CAT    1046
Glu Ile Phe Leu Pro Asn Asn Val Asp Gly Ser Pro Ala Ile Pro His
        225                 230                 235

GGG TCC AGA GTG AAG ATA CGT ATG GAC ACT CCA TCA GGT GTT AAG GAT    1094
Gly Ser Arg Val Lys Ile Arg Met Asp Thr Pro Ser Gly Val Lys Asp
    240                 245                 250

TCC ATT CCT GCT TGG ATC AAC TAC TCT TTA CAG CTT CCT GAT GAA ATT    1142
Ser Ile Pro Ala Trp Ile Asn Tyr Ser Leu Gln Leu Pro Asp Glu Ile
255                 260                 265                 270

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Pro Tyr Asn Gly Ile Tyr Tyr Asp Pro Pro Glu Glu Glu Arg Tyr Ile
                275                 280                 285

TTC CAA CAC CCA CGG CCA AAG AAA CCA AAG TCG CTG AGA ATA TAT GAA    1238
Phe Gln His Pro Arg Pro Lys Lys Pro Lys Ser Leu Arg Ile Tyr Glu
            290                 295                 300

TCT CAT ATT GGA ATG AGT AGT CCG GAG CCT AAA ATT AAC TCA TAC GTG    1286
Ser His Ile Gly Met Ser Ser Pro Glu Pro Lys Ile Asn Ser Tyr Val
        305                 310                 315

AAT TTT AGA GAT GAA GTT CTT CCT CGC ATA AAA AAG CTT GGG TAC AAT    1334
Asn Phe Arg Asp Glu Val Leu Pro Arg Ile Lys Lys Leu Gly Tyr Asn
    320                 325                 330

GCG GTG CAA ATT ATG GCT ATT CAA GAG CAT TCT TAT TAT GCT AGT TTT    1382
Ala Val Gln Ile Met Ala Ile Gln Glu His Ser Tyr Tyr Ala Ser Phe
335                 340                 345                 350

GGT TAT CAT GTC ACA AAT TTT TTN GCA CCA AGC AGC CGT TTT GGA ACN    1430
Gly Tyr His Val Thr Asn Phe Xaa Ala Pro Ser Ser Arg Phe Gly Thr
                355                 360                 365

CCC GAC GAC CTT AAG TCT TTG ATT GAT AAA GCT CAT GAG CTA GGA ATT    1478
Pro Asp Asp Leu Lys Ser Leu Ile Asp Lys Ala His Glu Leu Gly Ile
            370                 375                 380

GTT GTT CTC ATG GAC ATT GTT CAC AGC CAT GCA TCA AAT AAT ACT TTA    1526
Val Val Leu Met Asp Ile Val His Ser His Ala Ser Asn Asn Thr Leu
        385                 390                 395

GAT GGA CTG AAC ATG TTT GAC GGC ACA GAT AGT TGT TAC TTT CAC TCT    1574
Asp Gly Leu Asn Met Phe Asp Gly Thr Asp Ser Cys Tyr Phe His Ser
    400                 405                 410
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415 420 425 430

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450 455 460

ATG ATG TAT ACT CAC CAC GGA TTA TCG GTG GGA TTC ACT GGG AAC TAC 1766
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465 470 475

GAG GAA TAC TTT GGA CTC GCA ACT GAT GTG GAT GCT GTT GTG TAT CTG 1814
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480 485 490

ATG CTG GTC AAC GAT CTT ATT CAT GGG CTT TTC CCA GAT GCA ATT ACC 1862
Met Leu Val Asn Asp Leu Ile His Gly Leu Phe Pro Asp Ala Ile Thr
495 500 505 510

ATT GGT GAA GAT GTT AGC GGA ATG CCG ACA TTT TNT ATT CCC GTT CAA 1910
Ile Gly Glu Asp Val Ser Gly Met Pro Thr Phe Xaa Ile Pro Val Gln
515 520 525

GAT GGG GGT GTT GGC TTT GAC TAT CGG CTG CAT ATG GCA ATT GCT GAT 1958
Asp Gly Gly Val Gly Phe Asp Tyr Arg Leu His Met Ala Ile Ala Asp
530 535 540

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Lys Trp Ile Glu Leu Leu Lys Lys Arg Asp Glu Asp Trp Arg Val Gly
545 550 555

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560 565 570

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575 580 585 590

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Ala Phe Trp Leu Met Asp Lys Asp Met Tyr Asp Phe Met Ala Leu Asp
595 600 605

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Arg Pro Ser Thr Ser Leu Ile Asp Arg Gly Ile Ala Leu His Lys Met
610 615 620

ATT AGG CTT GTA ACT ATG GGA TTA GGA GGA GAA GGG TAC CTA AAT TTC 2246
Ile Arg Leu Val Thr Met Gly Leu Gly Gly Glu Gly Tyr Leu Asn Phe
625 630 635

ATG GGA AAT GAA TTC GGC CAC CCT GAG TGG ATT GAT TTC CCT AGG GCT 2294
Met Gly Asn Glu Phe Gly His Pro Glu Trp Ile Asp Phe Pro Arg Ala
640 645 650

GAA CAA CAC CTC TCT GAT GGC TCA GTA ATT CCC GGA AAC CAA TTC AGT 2342
Glu Gln His Leu Ser Asp Gly Ser Val Ile Pro Gly Asn Gln Phe Ser
655 660 665 670

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TAT GAT AAA TGC AGA CGG AGA TTT GAC CTG GGA GAT GCA GAA TAT TTA     2390
Tyr Asp Lys Cys Arg Arg Arg Phe Asp Leu Gly Asp Ala Glu Tyr Leu
                675                 680                 685

AGA TAC CGT GGG TTG CAA GAA TTT GAC CGG GCT ATG CAG TAT CTT GAA     2438
Arg Tyr Arg Gly Leu Gln Glu Phe Asp Arg Ala Met Gln Tyr Leu Glu
            690                 695                 700

GAT AAA TAT GAG TTT ATG ACT TCA GAA CAC CAG TTC ATA TCA CGA AAG     2486
Asp Lys Tyr Glu Phe Met Thr Ser Glu His Gln Phe Ile Ser Arg Lys
        705                 710                 715

GAT GAA GGA GAT AGG ATG ATT GTA TTT GAA AAA GGA AAC CTA GTT TTT     2534
Asp Glu Gly Asp Arg Met Ile Val Phe Glu Lys Gly Asn Leu Val Phe
    720                 725                 730

GTC TTT AAT TTT CAC TGG ACA AAA AGC TAT TCA GAC TAT CGC ATA GGC     2582
Val Phe Asn Phe His Trp Thr Lys Ser Tyr Ser Asp Tyr Arg Ile Gly
735                 740                 745                 750

TGC CTG AAG CCT GGA AAA TAC AAG GTT GCC TTG GAC TCA GAT GAT CCA     2630
Cys Leu Lys Pro Gly Lys Tyr Lys Val Ala Leu Asp Ser Asp Asp Pro
                755                 760                 765

CTT TTT GGT GGC TTC GGG AGA ATT GAT CAT AAT GCC GAA TAT TTC ACC     2678
Leu Phe Gly Gly Phe Gly Arg Ile Asp His Asn Ala Glu Tyr Phe Thr
            770                 775                 780

TTT GAA GGA TGG TAT GAT GAT CGT CCT CGT TCA ATT ATG GTG TAT GCA     2721
Phe Glu Gly Trp Tyr Asp Asp Arg Pro Arg Ser Ile Met Val Tyr Ala
        785                 790                 795

CCT AGT AGA ACA GCA GTG GTC TAT GCA CTA GTA GAC AAA GAA GAA GAA     2774
Pro Ser Arg Thr Ala Val Val Tyr Ala Leu Val Asp Lys Glu Glu Glu
    800                 805                 810

GAA GAA GAA GAA GTA GCA GTA GTA GAA GAA GTA GTA GTA GAA GAA GAA     2822
Glu Glu Glu Glu Val Ala Val Val Glu Glu Val Val Val Glu Glu Glu
815                 820                 825                 830

TGA ACGAA CTTGTGATCG CGTTGAAAGA TTTGAAGGCT ACATAGAGCT TCTTGACGTA     2880
***

TCTGGCAATA TTGCATCAGT CTTGGCGGAA TTTCATGTGA CAAAAGGTTT GCAATTCTTT   2940
CCACTATTAG TAGTGCAACG ATATACGCAG AGATGAAGTG CTGCACAAAC ATATGTAAAA   3000
TCGATGAATT TATGTCGAAT GCTGGGACGG GCTTCAGCAG GTTTTGCTTA GTGAGTTCTG   3060
TAAATTGTCA TCTC                                                     3074
```

## SEQ ID No. 2

Sequenced molecule: cDNA
Name: beII gene fragment (branching enzyme II) from
*Solanum tuberosum* (potato)
Length of sequence: 1393 bp

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T CTG CCA AAT AAT GTG GAT GGT TCT CCT GCA ATT CCT CAT GGG TCC AGA    49
  Leu Pro Asn Asn Val Asp Gly Ser Pro Ala Ile Pro His Gly Ser Arg
   1               5                  10                  15

GTG AAG ATA CGT ATG GAC ACT CCA TCA GGT GTT AAG GAT TCC ATT CCT      97
Val Lys Ile Arg Met Asp Thr Pro Ser Gly Val Lys Asp Ser Ile Pro
            20                  25                  30

GCT TGG ATC AAC TAC TCT TTA CAG CTT CCT GAT GAA ATT CCA TAT AAT     145
Ala Trp Ile Asn Tyr Ser Leu Gln Leu Pro Asp Glu Ile Pro Tyr Asn
        35                  40                  45

GGA ATA TAT TAT GAT CCA CCC GAA GAG GAG AGG TAT ATC TTC CAA CAC     193
Gly Ile Tyr Tyr Asp Pro Pro Glu Glu Glu Arg Tyr Ile Phe Gln His
    50                  55                  60

CCA CGG CCA AAG AAA CCA AAG TCG CTG AGA ATA TAT GAA TCT CAT ATT     241
Pro Arg Pro Lys Lys Pro Lys Ser Leu Arg Ile Tyr Glu Ser His Ile
65                  70                  75                  80

GGA ATG AGT AGT CCG GAG CCT AAA ATT AAC TCA TAC GTG AAT TTT AGA     289
Gly Met Ser Ser Pro Glu Pro Lys Ile Asn Ser Tyr Val Asn Phe Arg
                85                  90                  95

GAT GAA GTT CTT CCT CGC ATA AAA AAG CTT GGG TAC AAT GCG GTG CAA     337
Asp Glu Val Leu Pro Arg Ile Lys Lys Leu Gly Tyr Asn Ala Val Gln
            100                 105                 110

ATT ATG GCT ATT CAA GAG CAT TCT TAT TAT GCT AGT TTT GGT TAT CAT     385
Ile Met Ala Ile Gln Glu His Ser Tyr Tyr Ala Ser Phe Gly Tyr His
        115                 120                 125

GTC ACA AAT TTT TTN GCA CCA AGC AGC CGT TTT GGA ACN CCC GAC GAC     433
Val Thr Asn Phe Xaa Ala Pro Ser Ser Arg Phe Gly Thr Pro Asp Asp
    130                 135                 140

CTT AAG TCT TTG ATT GAT AAA GCT CAT GAG CTA GGA ATT GTT GTT CTC     481
Leu Lys Ser Leu Ile Asp Lys Ala His Glu Leu Gly Ile Val Val Leu
145                 150                 155                 160

ATG GAC ATT GTT CAC AGC CAT GCA TCA AAT AAT ACT TTA GAT GGA CTG     529
Met Asp Ile Val His Ser His Ala Ser Asn Asn Thr Leu Asp Gly Leu
                165                 170                 175

AAC ATG TTT GAC GGC ACA GAT AGT TGT TAC TTT CAC TCT GGA GCT CGT     577
Asn Met Phe Asp Gly Thr Asp Ser Cys Tyr Phe His Ser Gly Ala Arg
            180                 185                 190

GGT TAT CAT TGG ATG TGG GAT TCC CGC CTC TTT AAC TAT GGA AAC TGG     625
Gly Tyr His Trp Met Trp Asp Ser Arg Leu Phe Asn Tyr Gly Asn Trp
        195                 200                 205

GAG GTA CTT AGG TAT CTT CTC TCA AAT GCG AGA TGG TGG TTG GAT GAG     673
Glu Val Leu Arg Tyr Leu Leu Ser Asn Ala Arg Trp Trp Leu Asp Glu
    210                 215                 220
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TTC AAA TTT GAT GGA TTT AGA TTT GAT GGT GTG ACA TCA ATG ATG TAT 721
Phe Lys Phe Asp Gly Phe Arg Phe Asp Gly Val Thr Ser Met Met Tyr
225 230 235 240

ACT CAC CAC GGA TTA TCG GTG GGA TTC ACT GGG AAC TAC GAG GAA TAC 769
Thr His His Gly Leu Ser Val Gly Phe Thr Gly Asn Tyr Glu Glu Tyr
245 250 255

TTT GGA CTC GCA ACT GAT GTG GAT GCT GTT GTG TAT CTG ATG CTG GTC 817
Phe Gly Leu Ala Thr Asp Val Asp Ala Val Val Tyr Leu Met Leu Val
260 265 270

AAC GAT CTT ATT CAT GGG CTT TTC CCA GAT GCA ATT ACC ATT GGT GAA 865
Asn Asp Leu Ile His Gly Leu Phe Pro Asp Ala Ile Thr Ile Gly Glu
275 280 285

GAT GTT AGC GGA ATG CCG ACA TTT TNT ATT CCC GTT CAA GAT GGG GGT 913
Asp Val Ser Gly Met Pro Thr Phe Xaa Ile Pro Val Gln Asp Gly Gly
290 295 300

GTT GGC TTT GAC TAT CGG CTG CAT ATG GCA ATT GCT GAT AAA TGG ATT 961
Val Gly Phe Asp Tyr Arg Leu His Met Ala Ile Ala Asp Lys Trp Ile
305 310 315 320

GAG TTG CTC AAG AAA CGG GAT GAG GAT TGG AGA GTG GGT GAT ATT GTT 1009
Glu Leu Leu Lys Lys Arg Asp Glu Asp Trp Arg Val Gly Asp Ile Val
325 330 335

CAT ACA CTG ACA AAT AGA AGA TGG TCG GAA AAG TGT GTT TCA TAC GCT 1057
His Thr Leu Thr Asn Arg Arg Trp Ser Glu Lys Cys Val Ser Tyr Ala
340 345 350

GAA AGT CAT GAT CAA GCT CTA GTC GGT GAT AAA ACT ATA GCA TTC TGG 1105
Glu Ser His Asp Gln Ala Leu Val Gly Asp Lys Thr Ile Ala Phe Trp
355 360 365

CTG ATG GAC AAG GAT ATG TAT GAT TTT ATG GCT CTG GAT AGA CCN TCA 1153
Leu Met Asp Lys Asp Met Tyr Asp Phe Met Ala Leu Asp Arg Pro Ser
370 375 380

ACA TCA TTA ATA GAT CGT GGG ATA GCA TTG CAC AAG ATG ATT AGG CTT 1201
Thr Ser Leu Ile Asp Arg Gly Ile Ala Leu His Lys Met Ile Arg Leu
385 390 395 400

GTA ACT ATG GGA TTA GGA GGA GAA GGG TAC CTA AAT TTC ATG GGA AAT 1249
Val Thr Met Gly Leu Gly Gly Glu Gly Tyr Leu Asn Phe Met Gly Asn
405 410 415

GAA TTC GGC CAC CCT GAG TGG ATT GAT TTC CCT AGG GCT GAA CAA CAC 1297
Glu Phe Gly His Pro Glu Trp Ile Asp Phe Pro Arg Ala Glu Gln His
420 425 430

CTC TCT GAT GGC TCA GTA ATT CCC GGA AAC CAA TTC AGT TAT GAT AAA 1345
Leu Ser Asp Gly Ser Val Ile Pro Gly Asn Gln Phe Ser Tyr Asp Lys
435 440 445

TGC AGA CGG AGA TTT GAC CTG GGA GAT GCA GAA TAT TTA AGA TAC CGT 1393
Cys Arg Arg Arg Phe Asp Leu Gly Asp Ala Glu Tyr Leu Arg Tyr Arg
450 455 460

CLAIMS

1. An amino acid sequence of starch branching enzyme II (SBE II) comprising the amino acid sequence as shown in SEQ ID No. 1.

2. Fragments of the amino acid sequence of starch branching enzyme II (SBEII).

3. A fragment according to claim 2, having the amino acid sequence as shown in SEQ ID No. 2.

4. An isolated DNA sequence encoding starch branching enzyme II (SBE II) of potato comprising the nucleotide sequence as shown in SEQ ID No. 1 variants thereof resulting from the degeneracy of the genetic code.

5. Fragments of the isolated DNA sequence encoding starch branching enzyme II (SBEII) of potato.

6. A fragment according to claim 5, comprising the nucleotide sequence as shown in SEQ ID No. 2.

7. A vector comprising the whole or a functionally active part of the isolated DNA sequence claimed in any one of claims 4-6 and regulatory elements active in potato.

8. A vector according to claim 7, wherein the DNA sequence is in the antisense (reversed) orientation in relation to a promoter immediately upstream from the DNA sequence.

9. A process for the production of transgenic potatoes with either an increased or a decreased degree of branching of amylopectin starch, characterized in that it comprises the following steps:
a) transfer and incorporation of a vector according to claim 7 into the genome of a potato cell, and
b) regeneration of intact, whole plants from the transformed cells.

10. A process for the production of transgenic potatoes with a reduced degree of branching of amylopectin

starch, characterized in that it comprises the following steps:
a) transfer and incorporation of a vector according to claim 8 into the genome of a potato cell, and
b) regeneration of intact, whole plants from the transformed cells.

11. A process according to claim 10, wherein the vector also comprises an antisense construct of starch branching enzyme I (SBE I).

12. A process according to claims 10 or 11, wherein the vector also comprises an antisense construct of potato granule bound starch synthase II.

13. A process according to one or more of claims 10-12, wherein the vector also comprises an antisense construct of potato soluble starch synthases II and III.

14. A process according to one or more of claims 10-13, wherein the vector also comprises an antisense construct of potato starch disproportionating enzyme (D-enzyme).

15. A process according to one or more of claims 10-14, wherein the vector also comprises an antisense construct of potato starch debranching enzyme.

16. A transgenic potato obtainable by the process according to any one of claims 9-15.

17. Use of transgenic potatoes according to claim 16 for the production of starch.

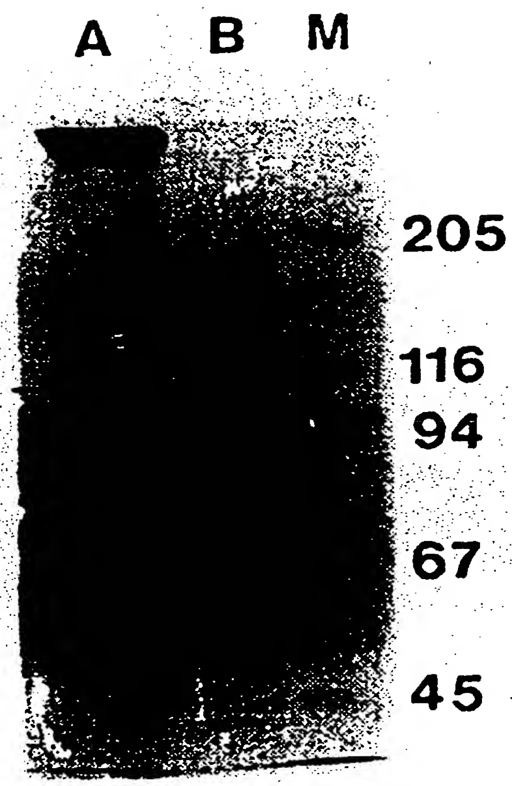


FIG 1

FIG. 2

Peptide 1. EFGVWEIFLPN

Peptide 2. HGLQEFDRA

Peptide 3. ENDGIAAKADE

Peptide 4. YEIDPEI/LTN

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 96/01558

## A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 9/10, C12N 15/82, A01H 5/06
According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, CA, BIOSIS, EMBL/GENBANK/DDBJ

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | WO 9504826 A1 (INSTITUT FüR GENBIOLOGISCHE FORSCHUNG BERLIN GMBH), 16 February 1995 (16.02.95), see abstract and claim 23 -- | 1-17 |
| X | WO 9214827 A1 (INSTITUT FüR GENBIOLOGISCHE FORSCHUNG BERLIN GMBH), 3 Sept 1992 (03.09.92), see page 5, line 1-7 and examples -- | 1-17 |
| A | SE 467160 B (AMYLOGENE HANDELSBOLAG), 1 June 1992 (01.06.92) -- -------- | 1-17 |

☐ Further documents are listed in the continuation of Box C.  ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 27 February 1997 | 01-03-1997 |
| Name and mailing address of the ISA<br>Swedish Patent Office<br>Box 5055, S-102 42 STOCKHOLM<br>Facsimile No. +46 8 666 02 86 | Authorized officer<br>Yvonne Sjösteen<br>Telephone No. +46 8 782 25 00 |

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/SE 96/01558

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
| --- | --- | --- | --- |
| WO-A1- 9504826 | 16/02/95 | AU-A- 7535294 | 28/02/95 |
| | | EP-A- 0713531 | 29/05/96 |
| | | CA-A- 2169174 | 16/02/95 |
| | | DE-A- 4327165 | 16/02/95 |
| | | HU-A- 73740 | 30/09/96 |
| | | HU-D- 9600285 | 00/00/00 |
| | | IL-D- 110583 | 00/00/00 |
| WO-A1- 9214827 | 03/09/92 | AU-B- 663072 | 28/09/95 |
| | | AU-A- 1226592 | 15/09/92 |
| | | CA-A- 2104123 | 14/08/92 |
| | | DE-A- 4104782 | 20/08/92 |
| | | EP-A 0571427 | 01/12/93 |
| | | HU-A- 65740 | 28/07/94 |
| SE-B- 467160 | 01/06/92 | AU-A- 9109791 | 22/07/92 |
| | | EP-A- 0563201 | 06/10/93 |
| | | PL-B- 169859 | 30/09/96 |
| | | SE-A- 9004095 | 01/06/92 |
| | | WO-A- 9211375 | 09/07/92 |